IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

ETZLER and ROBERTS

Application No.: 09/657,631

Filed: September 6, 2000

For: LNP, A PROTEIN INVOLVED IN THE INITIATION OF MYCORRHIZAL INFECTION IN PLANTS

Customer No.: 20350

Examiner: Baum, Stuart F.

Technology Center/Art Unit: 1638

Declaration of Biao Wu Ph.D.
Under 37 C.F.R. §1.132

Commissioner for Patents
Washington D.C. 20231
Sir:

I, Biao Wu, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true.

2. I was a Ph.D. student of Marilynn Etzler and conducted the following experiments as part of the requirement for completion of the Ph.D. degree. I have read and am familiar with the contents of the subject patent application.

3. In order to prove that mycorrhizal infection of a plant can be increased by transforming a plant with an expression cassette containing a plant promoter operably linked to a heterologous LNP polynucleotide as claimed in the above-identified patent application, the following experimental description is provided. The experiments reported herein were conducted by myself under the supervision of Dr. Etzler, and show that a heterologous LNP polynucleotide introduced on an expression cassette into a plant that is otherwise not capable of forming mycorrhizal associations, allows that plant to form mycorrhizal associations that are not possible in the absence of the LNP. Thus, the experiments demonstrate that heterologous LNP increases mycorrhizal associations of transgenic plants bearing the heterologous LNP.

4. **Transf rmation of *Arabidopsis thaliana* with *Dolichos biflorus* LNP permits external hyphae of germinated *Glomus intraradices* to attach to the root surface of the transformed *Arabidopsis* plants.**

5. *Arabidopsis thaliana,* which is typically a non-host for endomycorrhiza, was used to investigate the ability of heterologous LNP to promote formation of mycorrhizal associations in transgenic plants.

6. Two transgenic *Arabidopsis* lines DB4 and DB7, were created by transforming wild type *Arabidopsis* with plasmids that express the *Dolichos biflorus* LNP gene (SEQ ID NO:1). A third transgenic strain, CK4, was created by transforming wild type *Arabidopsis* with an empty vector plasmid. The DB4 and DB7 lines were tested for expression of *Dolichos biflorus* LNP (Db-LNP, SEQ ID NO:2) by immunoblotting, and were found to express Db-LNP at medium and low levels, respectively. As expected, neither the CK4 vector control line, nor wild type *Arabidopsis* showed any Db-LNP expression in these assays.

7. The seeds of the three transgenic *Arabidopsis* lines (DB4, DB7, and CK4) and wild type *Arabidopsis* were added to a soil mixture prepared by mixing an INVAM (International Culture Collection of Arbuscular & Vesicular-Arbuscular Mycorrhizal Fungi) culture containing the fungus, *Glomus intraradices,* with nine volumes of vermiculite. Sterilized *Lotus japonicus* seeds were placed around the *Arabidopsis* seeds. All the seeds were germinated, and six weeks later, the *Arabidopsis* roots were collected and stained in order to detect any mycorrhiza.

8. To collect the roots for examination, the Arabidopsis plants, together with soil, were immersed in water, and the roots were carefully separated from the soil and cut off from the plants. The roots were cleared in 2.5 % KOH for 60 minutes in a 90 °C water bath until the roots were translucent. The roots were then rinsed in water to dilute out the KOH and then most of the water was removed from the roots. The roots were then acidified in 1 % HCl for 60 minutes to enable staining and removed from the acid solution. The roots were immersed in 0.06 % trypan blue/20 % lactic acid/40 % glycerol overnight at room temperature with horizontal shaking. The roots were removed from the trypan blue, rinsed with water, and destained with 50 % glycerol for 4 hours with gentle shaking. The roots were then mounted in 50 % glycerol and observed under a microscope.

9. The results showed that while no arbuscules, vesicles or internal hyphae were found *inside* the roots of any transgenic line or wild type *Arabidopsis*, external hyphae were found attaching to the root surface of DB4 (Exhibit A, Figures A and B) and DB7 (Exhibit A, Figure C), but *not* to CK4 (Exhibit A, Figure D) nor wild type *Arabidopsis* plants. Thus, those plants expressing the transgenic *Dolichos biflorus* LNP gene were able to form mycorrhizal attachments with the hyphae of *Glomus intraradices* whereas the plants that did not express Db-LNP did not form these attachments.

10. Thus, these experiments demonstrate that overexpression of LNP in a transgenic plant is sufficient to increase mycorrhizal infection of that plant.

The Declarant has nothing further to say.

Dated: 12/10/03 By: Biao Wu
Biao Wu Ph.D.

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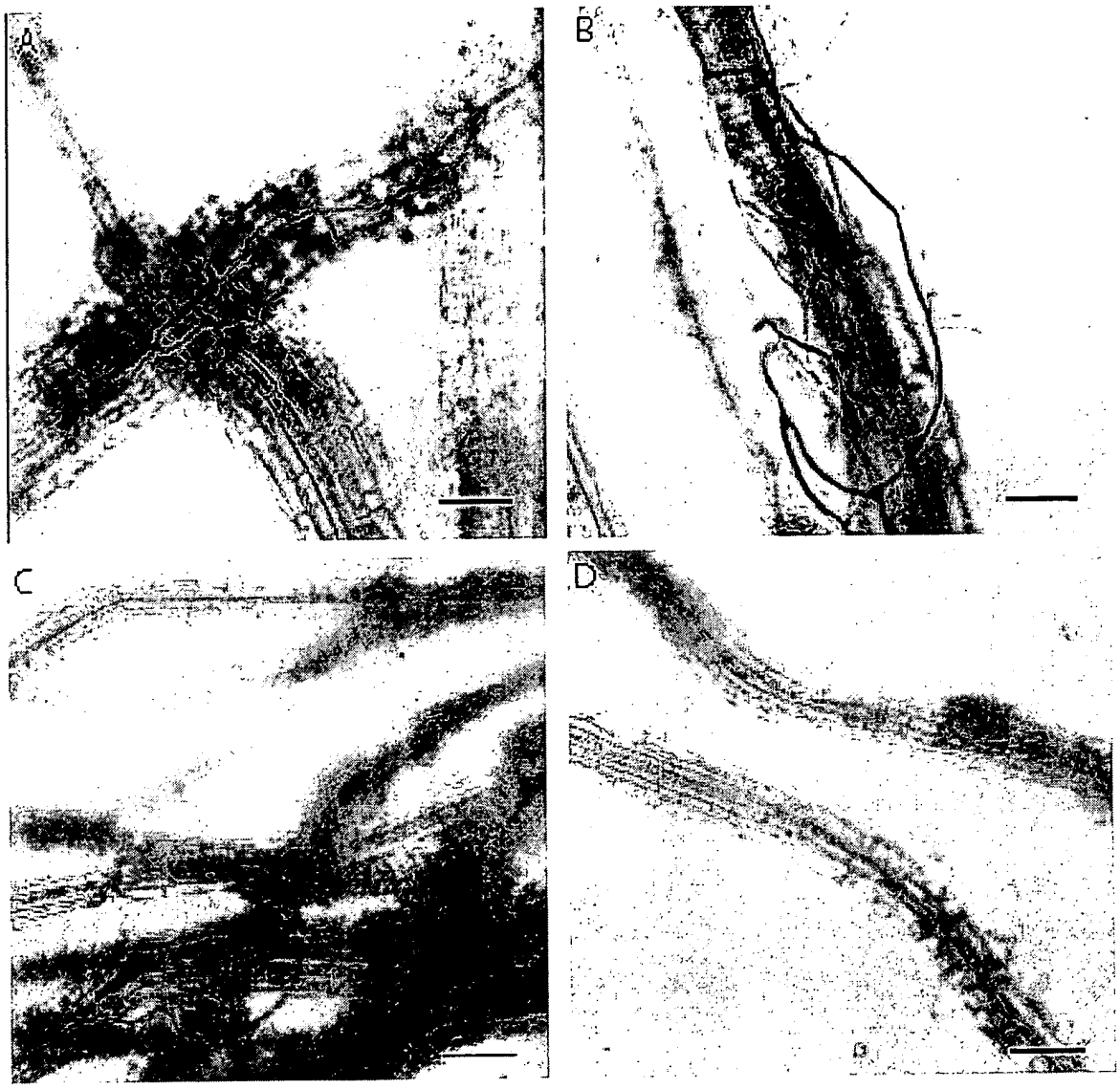
EXHIBIT A



Mycorrhizal hyphae attach to the root surface of transgenic *Arabidopsis* plants expressing Db-LNP.

Lotus japonicus and transgenic *Arabidopsis* seeds were germinated in a soil mixture inoculated with *Glomus intraradices* and grown for six weeks. Following growth, the roots were stained with trypan blue. After staining, hyphae were seen attached to the surface of the roots of DB4 (A and B) and DB7 (C) plants, but not to the roots of CK4 plants (D). Scale bar, 500μm.